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A Closer Look at Dark Toxicity of the Photosensitizer TMPyP in Bacteria

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ABSTRACT

Photodynamic inactivation of bacteria (PIB) is based on photosensitizers which absorb light and generate reactive oxygen species (ROS), killing cells via oxidation. PIB is evaluated by comparing viability with and without irradiation, where reduction of viability in the presence of the photosensitizer without irradiation is considered as dark toxicity. This effect is controversially discussed for photosensitizers like TMPyP (5,10,15,20-Tetrakis(1-methyl-4-pyridinio)porphyrin tetra(p-toluensulfonate). TMPyP shows a high absorption coefficient for blue light and a high yield of ROS production, especially singlet oxygen. *Escherichia coli* and *Bacillus atrophaeus* were incubated with TMPyP and irradiated with different light sources at low radiant exposures (μW per cm²), reflecting laboratory conditions of dark toxicity evaluation. Inactivation of *E. coli* occurs for blue light, while no effect was detectable for wavelengths >450 nm. Being more susceptible toward PIB, growth of *B. atrophaeus* is even reduced for light with emission >450 nm. Decreasing the light intensities to nW per cm² for *B. atrophaeus*, application of TMPyP still caused bacterial killing. Toxic effects of TMPyP disappeared after addition of histidine, quenching residual ROS. Our experiments demonstrate that the evaluation of dark toxicity of a powerful photosensitizer like TMPyP requires low light intensities and if necessary additional application of substances quenching any residual ROS.

INTRODUCTION

The WHO report on antimicrobial resistances published in 2014 stated that the antibiotic pipeline is nearly dry which will threat the people′s health even when they have only routine infections (1). This statement was reinforced 2017 by the publication of a global priority list of pathogens with antibiotic resistance that pose a severe problem to human health (2). This includes, for example, also methicillin-resistant *Staphylococcus aureus* (MRSA), one of the most important nosocomial and community-acquired pathogens (3–5). So far, MRSA has developed resistance not only against all β-lactam antibiotics but also against last resort antibiotics like vancomycin or linezolid (4). Further pathogens within the priority list of the WHO that are ranked as critical include carbapenem-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* (2). Therefore, apart from the investigation and development of new antibiotics, it is necessary to focus on alternative antibacterial approaches like photodynamic inactivation of bacteria (PIB). PIB is based on positively charged dye molecules that can attach to the negatively charged bacterial cell wall or are taken up by the cells (6–8). By exposure to visible light, the absorbed light energy is transferred to adjacent molecular oxygen and results in the generation of singlet oxygen. This highly reactive oxygen species attacks bacterial cell wall components and hence causes an irreversible oxidative damage of the microorganisms already during irradiation (9). In general, reactive oxygen species (ROS) constantly arise as metabolic by-products (10,11) or due to UV radiation and negatively affect macromolecules like DNA, lipids and proteins. Aerobic organisms evolved diverse response reactions and structures to protect themselves against oxidative stress (10). Enzymes like catalase and superoxide dismutase, carotenoid (12) and flavonoid pigments (13) or glutathione (14) act as quenchers or protective substances for ROS. Apart from that, one can also take advantage from the ROS' toxicity as conveyed by the mechanism of oxidative burst used in plant pathogen defense (15). Also PIB makes use of ROS by directly targeting the cell and using concentration levels above the ones that can be trapped by the organism's protective molecules. Additionally, the development of optical methods to monitor singlet oxygen production has provided valuable insights into the effects of PIB and unveiled quenching factors like solvent or dissolved solutes in the media (16).

Using porphyrins or phenothiazines as photosensitizers, the research in the field of PIB has escalated showing first positive results regarding inactivation of different pathogens (17–23). The porphyrin TMPyP is a potent photosensitizer that preferentially generates singlet oxygen upon light irradiation and is widely used in PIB (24–27). Besides from attaching to cell walls, it can also bind to DNA *in vitro* (28). However, experiments using eukaryotic cells showed partly the presence of a high toxicity against these cells even in the absence of light. Kassab observed a dark toxicity toward HepG2 cells especially in the presence of a cytostatic drug in combination with TMPyP (29).

Dark toxicity was also described for various prokaryotic organisms like *Pseudomonas aerugionosa* (30), *E. coli* (31), *Bacillus cereus* (32) and *Staphylococcus aureus* (33) by several authors. However, experiments with *B. atrophaeus* did not provide clear results (32). Furthermore, dark toxicity seemed to depend on photosensitizer concentration (31) and mode of life (biofilms, planktonic cells) (30,33) In contrast, other authors

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observed no dark toxicity of TMPyP for bacterial cells (34–36). It is important to notice that most of the studies differ in their experimental setup hampering a direct comparison of the obtained data. To further clarify the situation, we investigated possible influences like wavelength, incubation time and irradiation intensity in dark controls for the model organisms *Escherichia coli* and *Bacillus atrophaeus*.

MATERIALS AND METHODS

Photosensitizer. The photosensitizer TMPyP (5,10,15,20-Tetrakis(1-methyl-4-pyridinio)-porphyrin tetra(p-toluenesulfonate) used for the experiments was purchased by Sigma-Aldrich Co. LLC with a minimum dye content of 97%. A solution was prepared using sterile, highly pure and filtered water (conductivity > 18 Ω), called $H_2O_{Millipore}$. The spectra of the photosensitizer were measured with the photometer mentioned below. The quantum yield of the photosensitizer is $\Phi_\Delta = 0.77$ (37).

Cultivation of bacteria. The assays were performed using *Escherichia coli* K12 (ATCC® 25922™) or *Bacillus atrophaeus* (ATCC® 9372™) cells. As preculture, the bacteria were grown on LB agar plates and single colonies were used for each experiment. The cells were grown overnight for 14 h in LB medium without glucose (38). After cultivation, the cells were harvested by centrifugation at 3500 ***g*** for 7 min and washed two times using either $H_2O_{Millipore}$ or histidine solution (1 or 5 mM; pH 7.0). Histidine as scavenger was used only with *B. atrophaeus* at 1 and 5 mM. Optical density was measured at $\lambda = 600$ nm via photometer (10 × 4 × 45 mm cuvettes, Sarstedt AG & Co.; V-3000PC Spectrophotometer, VWR) and adjusted to an $OD_{600} = 0.60–0.68$ (corresponding to about 10^8 bacteria per mL) by diluting with the respective solvent.

Preparation of the assays. A stock solution of the photosensitizer of 500 μM was prepared in $H_2O_{Millipore}$. Experiments were conducted in 96-well microtiter plates (Corning Costar, Corning, NY) using final concentrations of 5, 10, 50, 100 and 250 μM of the photosensitizer. As a control, a reaction without photosensitizer was performed using 25 μL $H_2O_{Millipore}$ instead of photosensitizer solution. In order to exclude any further effects of illumination, controls in almost darkness (<10 nW cm^{-2} light intensity, referring to the limit of detection of the sensor) were performed in the same way as the illuminated assays, yet with (250 μM) and without photosensitizer. These controls are hereafter addressed as dark control. In the case of *B. atrophaeus,* dark controls were performed with all photosensitizer concentrations yet without any additional light source. Final reaction mix was obtained by mixing of 25 μL bacterial suspension (as described above) and 25 μL of TMPyP in the appropriate concentration. After incubation for 1 h, serial dilutions up to 10^{-7} were prepared in LB medium. Dilutions from 10^{-7} to 10^{-1} were plated on Mueller-Hinton agar (39) using drop plate method (40) by plating three times 20 μL of each dilution step. After incubation at 37°C overnight, colonies were counted. Due to this experimental procedure, the detection limit was 166 CFU mL^{-1}. The data were obtained by at least three independent replicates. Colonies were counted and then converted into CFU per mL. Mean and standard deviation were calculated and visualized as bar charts using SigmaPlot. As biologically relevant, a reduction of three magnitudes of the decadal logarithm (≥99.9%) was defined, and five magnitudes of the decadal logarithm (≥99.999%) correspond to an effective disinfection (41). To evaluate long-term incubation effects of TMPyP on bacteria without light, the experimental procedure was performed as described in almost darkness and incubated for 1, 3, 6, 12, 18 and 24 h. Serial dilutions were prepared and plated as specified above.

Illumination conditions. Preparation of the reaction mix, serial dilutions and plating (time requirement about 10 min) were performed using different illuminations. Blue light conditions were generated with a LED at 405 nm (OSRAM GmbH, Germany). For yellow and red irradiation, a 15-W light bulb (OSRAM GmbH) and appropriate filter devices (INAKTIN 2012 and 2042, Kindermann GmbH, Eibelstadt, Germany) were used. Green light conditions were created by an 11-W green illumination device (OSRAM GmbH). To achieve a consistent distribution of light, a ground glass was placed between the light bulb or appropriate filter and the microtiter plates. All incubations were performed in a dark room without any additional source of light. For each experiment, the light intensity in the experimental area was measured using a photosensor PD300-SH (Ophir Optronics GmbH, Nienburg, Germany) and as display device the Nova-display (Ophir Optronics GmbH, Nienburg, Germany). Measured output light intensity values in the corresponding experimental area are displayed in Tables 1 and 2.

Table 1. Light intensity of the different illumination devices in the experimental area.

Experimental setup	Measured value [μW per cm^2]
Blue LED	141.1 ± 0.1
Red filter	157.2 ± 0.4
Yellow filter	185.0 ± 0.5
Green light	152.1 ± 0.5

Table 2. Intensity of ambient light using different darkening modes in the experimental area of the laboratory.

Darkening mode	Measured value range [μW per cm^2]
Dark laboratory	3 ± 1
Dimmed laboratory	55 ± 6
No direct light	132 ± 3

Likewise, experiments in controlled laboratory environments were conducted using *E. coli* cells. The light intensity of residual ambient light (Table 2) was adjusted with different darkening modes of the laboratory on a sunny, cloudless day without any other light source. Experiments under mentioned conditions were conducted in the same manner as experiments for different illumination conditions. The condition "dark laboratory" can be compared to a night of a full moon while "dimmed laboratory" rather corresponds to a candle in around 50 cm distance. "No direct light" conditions correspond to twilight with the sun settled under the horizon.

Emission spectra of the light sources were recorded by means of a monochromator with a CCD detection system (SPEX 232; HORIBA Jobin Yvon, Longjumeau Cedex, France).

RESULTS

TMPyP shows a maximum absorption (Soret band) at $\lambda_{max} = 421$ nm. Furthermore, four minor peaks are present around $\lambda = 520$ nm, 558 nm, 584 nm and 638 nm (Fig. 1a) representing the Q bands. Figure 1b shows the spectral photon distribution of the different illumination devices and the ambient light in the laboratory.

To compare the impact of different light sources applied, the absorption cross section of TMPyP was multiplied with the respective emission spectrum of each light source and integrated (42). Experimentally, a TMPyP concentration of 50 μM and a sample thickness of 2 mm (bacteria suspension in the well plate) was used. About 65% of the applied photons were absorbed in TMPyP for the blue LED spectrum, 15% for the indoor spectrum (none closed, no direct sunlight, see Table 2), 3% for the green spectrum and <2% for the yellow or red spectrum.

Effects of TMPyP on *E. coli* cells

Preliminary experiments for *E. coli* with and without TMPyP under common laboratory conditions indicated strong fluctuations (corresponding to ≥2 orders of $\log_{10}$) of the viable cells in dark controls performed under standard laboratory conditions (data not shown).

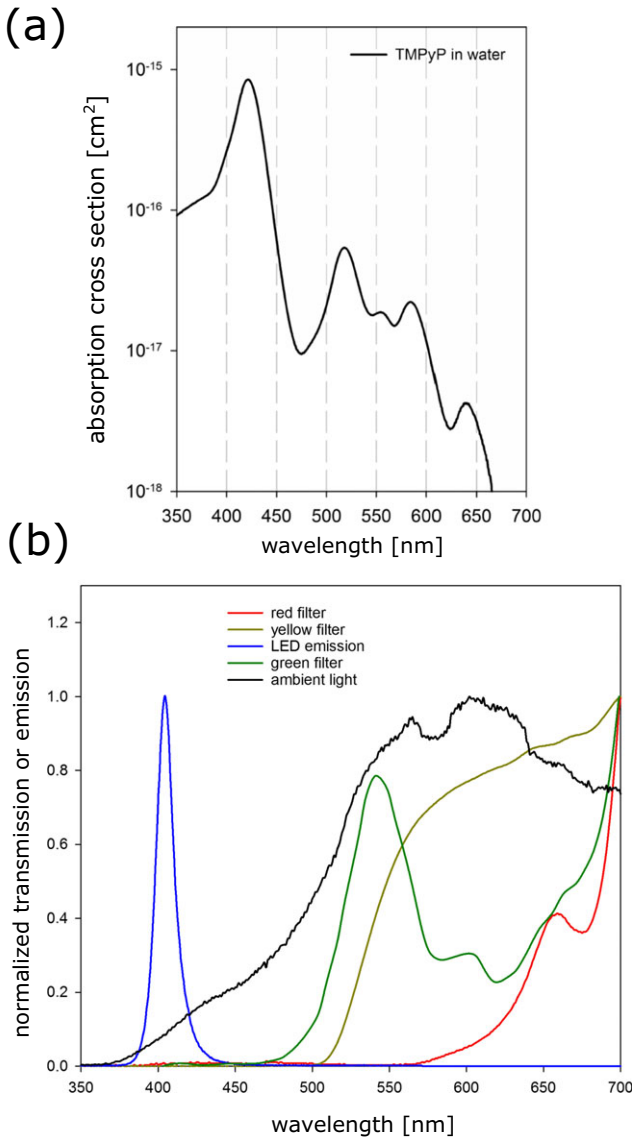

Figure 1. (a) Absorption curve of TMPyP; (b) normalized transmission or emission of the used illumination devices.

Effects of different spectral distributions. To investigate whether TMPyP exhibits a real dark toxicity as known from eukaryotic cells, experiments were performed using different conditions of illumination. Figure 2 shows the results of the *E. coli* cells washed in $H_2O_{Millipore}$. Corresponding to the definitions of hand hygiene, a detrimental effect was observed only for blue light conditions, but already at low concentrations of TMPyP. A biologically relevant effect occurred even at a concentration of 5 μM with a reduction of ≥4 $\log_{10}$. For 10, 50 and 100 μM, the reduction reached the lower limit of detection (≥6 $\log_{10}$). In the highest concentration (250 μM), a decrease of five orders of magnitude in viability was observed. Within experimental accuracy, green, yellow and red light showed only little, nonbiologically relevant effects with a reduction of less than one $\log_{10}$. Therefore, only blue illumination conditions led to a reduction exceeding one order of decadal logarithm.

Effect of different intensities of ambient light. Most importantly, all dark controls showed no reduction of more than 1 $\log_{10}$ even at an incubation time for 1 h (Fig. 3). PIB assays conducted at around 3 μW cm^{-2} (Fig. 3, Table 2) revealed no relevant reduction of cell viability (≤1 $\log_{10}$). When the reaction mix in $H_2O_{Millipore}$ was exposed to about 55 μW cm^{-2} (Fig. 3, Table 2), cell viability exhibits a reduction between one and two $\log_{10}$ for 50 and 100 μM of TMPyP.

Application of about 132 μW cm^{-2} (Fig. 3, Table 2) showed a reduction of ≥3 $\log_{10}$ for cells washed in $H_2O_{Millipore}$ and concentrations of 5, 10, 50 and 100 μM, whereas for 250 μM, TMPyP no reduction was observed.

Effect of different incubation times. Escherichia coli cells were incubated with the maximum concentration of TMPyP (250 μM) and incubation times of 1, 3, 6, 12, 18 and 24 h under dark conditions without irradiation (<10 nW cm^{-2}). No significant differences in the survival rates compared to controls without TMPyP were obtained, indicating that this parameter is of no significance for the tested organisms (Fig. 4).

Effects of TMPyP on *Bacillus atrophaeus*

Experiments using *B. atrophaeus* were carried out under different illumination conditions. For an irradiation at 405 nm, the lower limit of detection was reached for all concentrations (Fig. 5a). Application of red light caused a significant reduction of at least >2.5 $\log_{10}$ at TMPyP concentrations of 50, 100 or 250 μM, whereas green and yellow illuminations caused 2–3 $\log_{10}$ at 100 μM of TMPyP. The addition of the singlet oxygen quencher histidine (1 mM) remarkably reduced the bacteria killing effects when using the same illumination conditions (Fig. 5a and b).

When using ambient light in the laboratory, even the low light intensity condition caused reduction of bacteria viability of up to 3 $\log_{10}$ (Fig. 6). However, this effect could be abolished by adding singlet oxygen quencher histidine at concentrations of 1–5 mM.

DISCUSSION

Dark toxicity of TMPyP against bacteria has been under discussion for a long time. It was not the purpose of our study to reproduce the published studies with TMPyP as photosensitizer. TMPyP was investigated for antimicrobial PDT against various organisms using various experimental conditions in which concentration and incubation time of TMPyP could play a major role for dark toxicity. Thus, we concluded to cover the broad range of published concentrations (1–366 μM) (6,7,24,25,27,29–36,43–45) using TMPyP concentrations in our study from 0 to 250 μM. Incubation time in the present experiments was 1 h because most of incubation times published in literature ranged from a few minutes up to a few hours (6,7,24,25,27,29–36,43–45).

In the presented experiments, *E. coli* showed no decrease of cell viability in dark controls (<10 nW cm^{-2}) or when experiments were conducted with green, yellow or red light, even after 60-min incubation with 250 μM of TMPyP (Fig. 2). With increasing light intensity (Tables 1 and 2), the bacterial reduction rate increases from about 0.1 $\log_{10}$ to 5 $\log_{10}$ (Figs. 2 and 3). PIB effect was maximal for the blue light and less pronounced

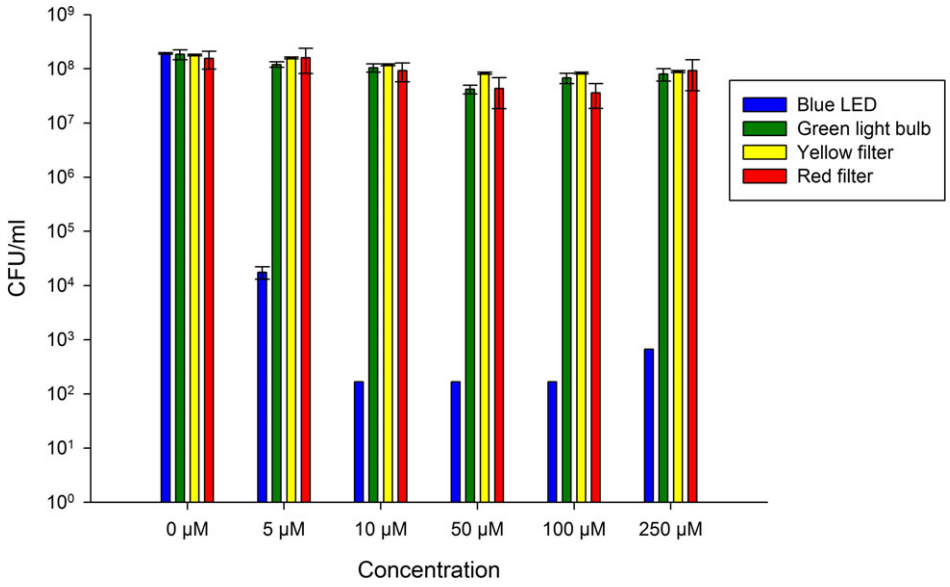

Figure 2. Survival rate displayed as CFU per mL for *E. coli* cells irradiated with blue, green, yellow and red light. Incubation time was 1 h, irradiation time 10 min. *Y*-axis indicates CFU per mL; *x*-axis indicates the concentrations of TMPyP. Error bars indicate experimental accuracy calculated as standard error.

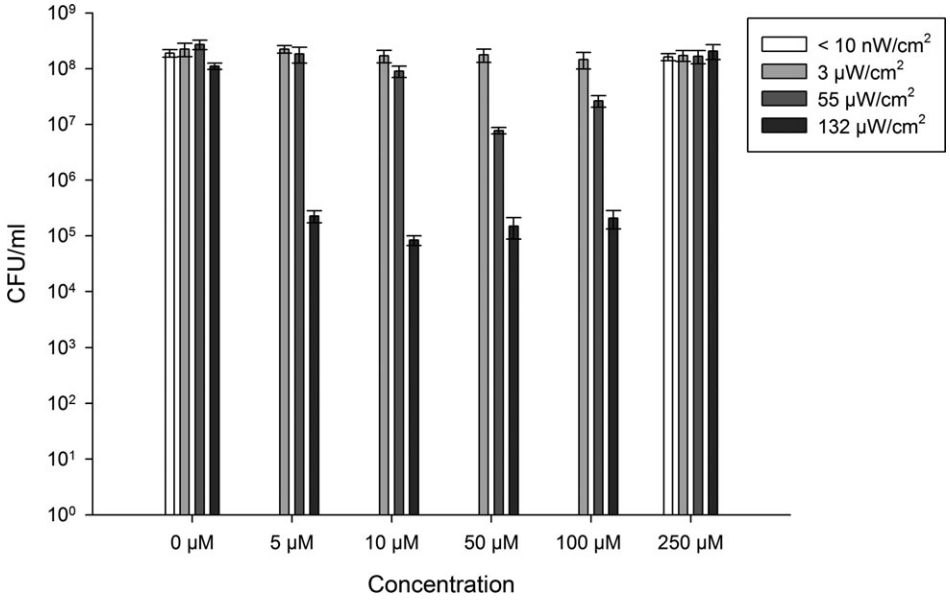

Figure 3. Survival rate displayed as CFU per mL for experiments performed with *E. coli* cells at different light intensities. Incubation time was 1 h, irradiation time 10 min. *Y*-axis indicates CFU per mL; *x*-axis indicates the concentrations of the photosensitizer. Error bars indicate experimental accuracy calculated as standard error. Values for <10 nW cm^{-2} were only determined for 0 and 250 µM TMPyP.

for ambient light, which is comparable to the percentage of absorbed photons in TMPyP. Almost no PIB effect is measured for green, yellow and red illumination. All three illumination spectra show only a small percentage of absorbed photons.

When keeping the bacteria under dark conditions (<10 nW cm^{-2}), no dark toxicity in *E. coli* was detected within experimental accuracy even for a very high concentration of TMPyP (250 µM) and long incubation times of up to 24 h (Fig. 4). Thus, it is assumed that dark toxicity of TMPyP, against the studied bacteria at least, can be attributed to PIB effects which are caused by any residual light in a laboratory.

It is well known that Gram-positive bacteria are more susceptible to PIB than Gram-negative bacteria (34). Also in the present study, *B. atrophaeus* showed growth inhibition at significant lower light intensities. Even in dark controls (<10 nW cm^{-2}), a reduction of viability of two to three orders of magnitude was obtained. However, this is not an effect of dark toxicity as could be demonstrated in parallel experiments in the presence of the scavenger histidine. It remains somewhat puzzling that *B. atrophaeus* cells showed a higher reduction under red light conditions as for yellow light conditions, which was quite unexpected especially when considering the

absorbed photons, therefore requiring further research in the future.

So far, studies on the dark toxicity of TMPyP describe either the existence or the absence of a dark toxicity: As an example, for both Gram-positive and Gram-negative bacteria, a dark toxicity was reported (30,32,33). In 2006, Donnelly *et al.* (30) concluded that TMPyP is toxic toward *Pseudomonas aeruginosa* cells isolated from clinical samples of cystic fibrosis patients. Nevertheless, all these studies lack a definition of dark control concerning the radiant exposure of residual light. In addition, there are further reports which show a concentration-dependent bactericidal effect of TMPyP against *E. coli* without irradiation (26). Studies on MRSA, *Bacillus cereus* and *Bacillus anthracis* reported a toxicity of TMPyP without light application although the effects were not significant (32,33).

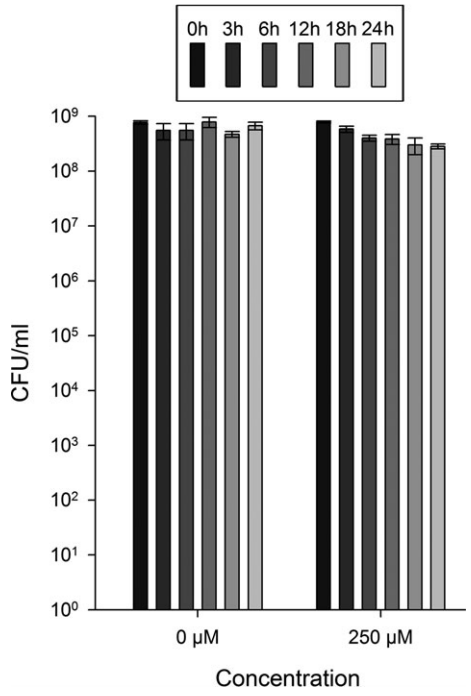

Figure 4. Survival rate displayed as CFUper mL for experiments performed with *E. coli* cells and 250 µM photosensitizer for incubation times of 0, 3, 6, 12, 18 or 24 h. *Y*-axis indicates CFU per mL; *x*-axis indicates the concentrations of photosensitizer. Error bars indicate experimental accuracy calculated as standard error. Experiments were conducted at <10 nW cm^{-2}.

On the contrary, a number of studies observed no dark toxicity of TMPyP. In 2010, Cassidy *et al.* demonstrated that there is no loss of viability in the presence of TMPyP and in the absence of light in experiments performed with *S. aureus* and *P. aeruginosa* (35). Different *E. coli* strains exhibit no dark toxicity of the tested concentrations (25,26,45).

The present work offers two possible explanations for these controversial results: we were able to demonstrate that extremely low light intensity already results in toxic effects on bacteria, even at quite low concentrations of TMPyP. In addition, this effect depends on the wavelength of the light source used. In particular for Gram-negative bacteria, an illumination with blue light, corresponding to the Soret band, was highly effective, while excitation with green, yellow and red light, interlinked with the Q bands, was ineffective. In contrast, Gram-positive bacteria showed a slight sensitivity after illumination at higher wavelength, although TMPyP concentrations of at least 50 µM were necessary.

The reduced efficiency of inactivation at concentrations of 250 µM is explainable due to the high extinction of TMPyP, as no blue light reaches the bottom of the well plate in our experimental setup (thickness of bacteria suspension in the well plate is about 2 mm). Wavelengths of 520 nm and lower still allow light to pass through to the bottom of the bacterial suspension at 250 µM.

Nitzan and Ashkenazi (45) described a killing effect toward *E. coli* and *Acinetobacter baumannii* cells at wavelengths within the range of the Q bands of TMPyP. However, for red and green

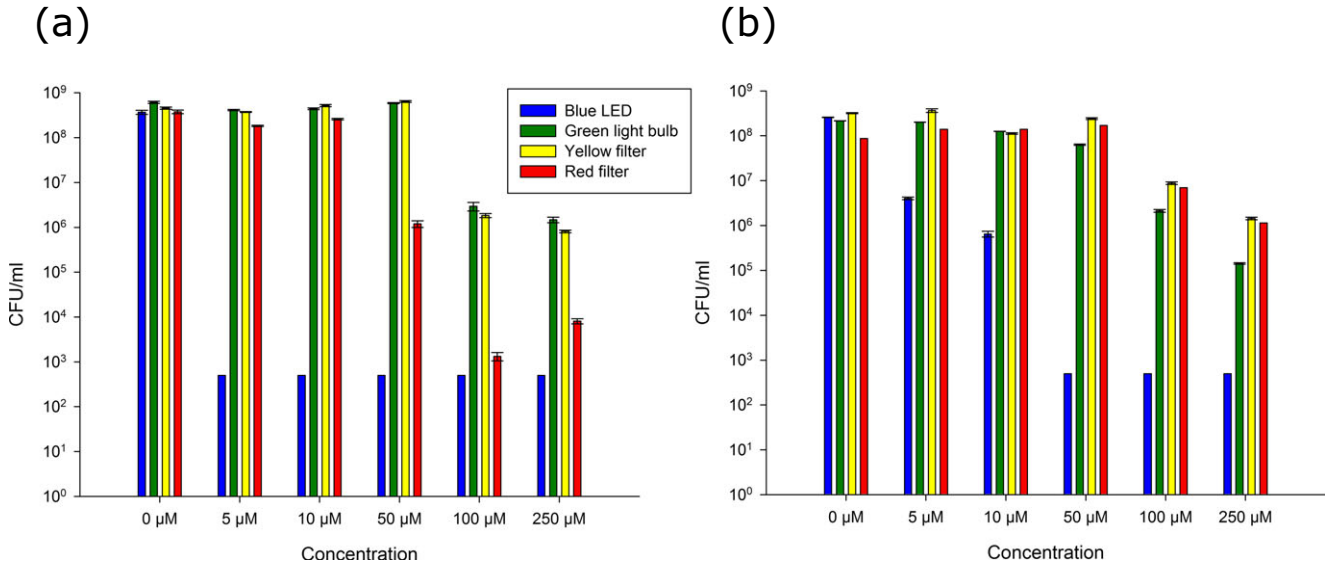

Figure 5. Survival rate displayed as CFU per mL for *B. atrophaeus* cells irradiated with blue, green, yellow and red light while for (a) cells were washed in $H_2O_{Millipore}$ and (b) in the presence of 1 mM histidine. Incubation time was 1 h, irradiation time 10 min. *Y*-axes indicate CFU per mL; *x*-axes indicate the various concentrations; error bars indicate the experimental accuracy calculated as standard error.

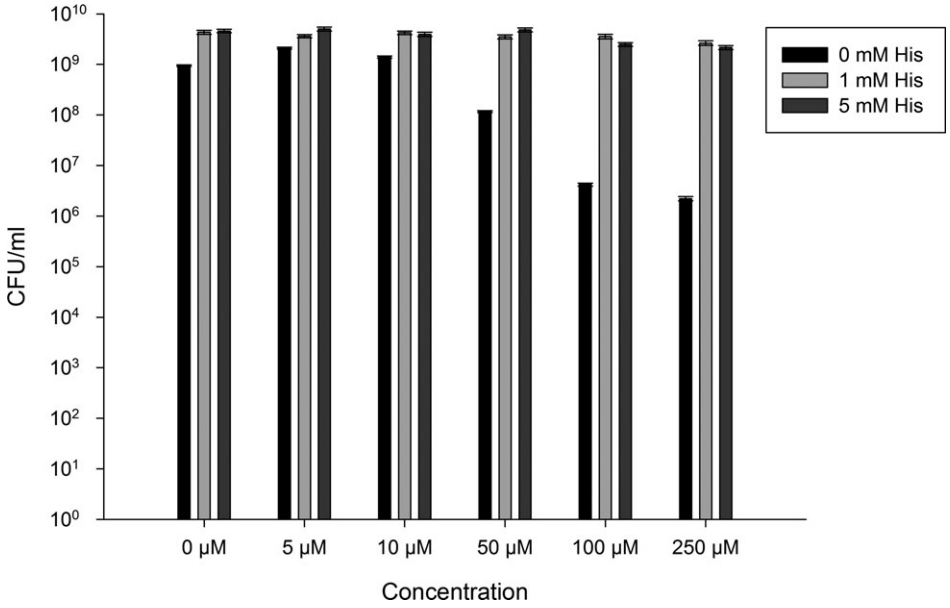

Figure 6. Survival rates displayed as CFU per mL for *B. atrophaeus* cells at <10 nW cm^{-2} without histidine, 1 or 5 mM histidine. Incubation time was 1 h, irradiation time 10 min. *Y*-axis indicates CFU per mL; *x*-axis indicates the various concentrations; error bars indicate the experimental accuracy calculated as standard error.

light, they needed up to 20 times higher radiant exposures for the inactivation than required for blue light (45). In contrast, our experiments showed no inactivation of *E. coli* when the experiments were carried out under green, yellow or red light. However, in the present study, the applied radiant exposure was significantly less as compared to the one used in the work of Nitzan and Ashkenazi for green and red light (45). This demonstrates that photons are absorbed to a much smaller extent within the Q bands leading to reduced amounts of produced ROS and therefore inactivation. Interestingly, in experiments with *B. atrophaeus,* the reduced radiant exposure, even with red, yellow and green light, resulted in significant growth inhibitions, demonstrating again the increased susceptibility of Gram-positive bacteria to ROS as described also in former studies (31,34).

The outcome of this study may also be helpful in research concerning cancer therapy. The characteristic structure of this porphyrin allows the molecule to bind G-quadruplex structures of DNA. Hurley *et al.* figured out that the c-MYC oncogene can be silenced using TMPyP as it distorts the quadruplex of a purine-rich element (NHE III$_1$) (46,47). However, depending on the quadruplex sequence, TMPyP reveals stabilizing or distorting properties (46,48,49). Additionally, TMPyP binds also telomeric G-quadruplexes which are often correlated with most cancer types (50,51). It seems to be advantageous to consider the herein presented results when researching the effects of TMPyP as a drug for cancer treatment. Furthermore, the effect of distortion might be partly deduced from residual light leading to a production of ROS and consequently to the destruction of such quadruplex structures.

CONCLUSION

Taking a glance at the different parameters that were chosen for the studies mentioned above it becomes obvious that experiments were conducted in an incomparably diverse manner that leads to diverging results. Investigations differ in experimental conditions such as used strains, applied radiant exposures, spectral emission of light sources, incubation time and photosensitizer concentration. Experiments in former studies used TMPyP concentrations ranging from 1 to 366 µM or incubation times from 30 s up to 24 h. It is remarkable that all published studies so far used higher radiant exposures compared to the present study, which shows very efficient reduction of bacterial viability even at low intensity of blue light. This supports the possibility that probably in the former studies, residual light during experimental work might have affected the measurement of dark toxicity. This is based on our findings with defined radiant exposures of residual light: cell viability is increasingly affected with increasing light intensity of residual light.

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